AD_____

Award Number: DAMD17-99-1-9183

TITLE: Antibody - Pretargeted Cytokine Therapy of Cancer

PRINCIPAL INVESTIGATOR: Louis M. Weiner, M.D.

CONTRACTING ORGANIZATION: Fox Chase Cancer Center
Philadelphia, Pennsylvania 19111

REPORT DATE: May 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 074-0188*



| 1. AGENCY USE ONLY (Leave blank) | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | May 2002 | Annual (15 Apr 01 - 14 Apr 02) |

**4. TITLE AND SUBTITLE**
Antibody - Pretargeted Cytokine Therapy of Cancer

**5. FUNDING NUMBERS**
DAMD17-99-1-9183

**6. AUTHOR(S)**
Louis M. Weiner, M.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
Fox Chase Cancer Center
Philadelphia, Pennsylvania 19111

E-Mail: LM_Weiner@fccc.edu

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**12b. DISTRIBUTION CODE**

**13. Abstract** *(Maximum 200 words)* *(abstract should contain no proprietary or confidential information)*

We hypothesize that the selective accumulation of systemically administered cytokines at tumor sites can alter tumor microenvironments to favor the induction of anti-tumor immune responses. We further hypothesize that this can be accomplished by pre-targeting tumors with antibody-streptavidin immunoconjugates and then administering biotinylated cytokines. The purpose of this research program is to identify antibody-pretargeted cytokine therapy strategies that lead to tumor-selective cytokine accumulation, the development of host inflammatory cell infiltrates in tumor, and the induction of tumor-specific immunity. The ultimate goal of this research is to identify candidate strategies for clinical development, alone or in combination with tumor vaccines. We have made significant progress toward achieving these goals. Because biodistribution results did not suggest that the previously used streptavidin-biotin system would yield therapeutic results we have focused efforts in the past year on developing new systems employing bispecific minibodies that contain anti-tumor binding domains and domains that bind to a metal chelate that can serve as a universal acceptor for metal-conjugated cytokines.

| 14. SUBJECT TERMS | 15. NUMBER OF PAGES |
|---|---|
| breast cancer, antibody, pretargeting, cytokine | 15 |
| | 16. PRICE CODE |

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

# Table of Contents

## INTRODUCTION

We hypothesize that the selective accumulation of systemically administered cytokines at tumor sites can alter tumor microenvironments to favor the induction of anti-tumor immune responses. We further hypothesize that this can be accomplished by pre-targeting tumors with antibody-streptavidin immunoconjugates and then administering biotinylated cytokines. The purpose of this research program is to identify antibody-pretargeted cytokine therapy strategies that lead to tumor-selective cytokine accumulation, the development of host inflammatory cell infiltrates in tumor, and the induction of tumor-specific immunity. The ultimate goal of this research is to identify candidate strategies for clinical development, alone or in combination with tumor vaccines.

# BODY

## Technical Objectives

1. To determine the conditions required to selectively pretarget streptavidin to human tumor xenografts growing in immunodeficient scid mice.
2. To determine conditions required for the selective accumulation of intravenously-administered biotinylated proteins and peptides to antibody-streptavidin pretargeted human tumor xenografts growing in immunodeficient scid mice.
3. To examine the host cellular infiltrate at tumor sites in mice following therapy with cytokines pretargeted to tumors by streptavidin-conjugated antibodies.
4. To examine the growth properties of tumors in mice treated with antibody-pretargeted cytokines.

## Work Accomplished

We previously achieved objectives 1 and 2. Careful analysis of biodistribution results using antibody-strepavidin tumor targeting and biotin-IL-2 delivery to the pretargeted tumors (Specific Aim 2) did not predict for therapeutic success due to innate deficiencies in the tumor targeting properties of the antibody-streptavidin conjugates. Accordingly, we devised an alternate strategy that utilizes a related pretargeting concept that addresses many of the deficiencies of the previously-employed approach.

NR-LU-10 – streptavidin immunoconjugate was obtained from NEORx Corporation, and was shown to bind by flow cytometry to HT-29 cells that overexpress Ep-CAM antigen (not shown). IL-2 was labeled through its carboxy-terminal cysteine according to manufacturer's instructions (Pierce). The biotinylated IL-2 was purified and removed over an avidin column (Pierce) and eluted with 100 mM glycine, pH3.0.

### Characterization of Biotinylated IL-2(Bt-IL-2)

The binding of IL-2 species to the IL-2 dependent NK92 cell line known to express the high affinity IL-2 receptor was measured by flow cytometric analysis. The results are depicted in the table below. The biotinylated species exhibited significant, but reduced binding to the IL-2 receptor. Bt-IL-2 bound to tumor cells coated with NRLU-10 – streptavidin. Half-maximal stimulation of T cell proliferation occurred at 0.005 – 0.01 nM IL-2, and at 0.1 – 0.5 nM biotinylated IL-2, respectively. Biotinylation reduced the T-cell proliferative effects of IL-2, but had relatively little impact on the ability of this cytokine to activate lymphocytes for tumor lysis. However, the immunoconjugate did not promote significant ADCC, presumably because the bulky streptavidin conjugation sites interfere with Fc domain interactions with lymphocyte Fc receptors.

Initial in vivo studies showed that best tumor to organ ratios were achieved at 30 h following injection with NRLU1—streptavidin. NRLU10-streptavidin then was injected into cohorts of scid mice bearing HT-29 subcutaneous xenografts (250 mg) followed by

the injection of a clearing agent 24 h later to remove any unbound NRLU10-streptavidin from the bloodstream. Thirty hours post-antibody injection, $^{125}$I-labeled Bt-IL-2 was injected into the mice. Tumors and normal organs were assayed for radioactive content and results used to calculate % injected dose per gram of tumor or organ, and to calculate tumor: normal organ ratios. $^{125}$I-Bt-IL-2 was successfully targeted to tumor sites as compared to pretargeted $^{125}$I-IL-2 or either of these molecules administered alone, but tumor to normal organ ratios were not high enough to predict that the amount of Bt-IL-2 delivered to tumor site would be sufficient to induce tumor regression. However, these initial results demonstrate that the concept of antibody-pretargeted cytokine therapy continues to have merit and is worth pursuing if an improved pretargeting system can be identified, produced and implemented.

In the past year our efforts have concentrated on the construction of bispecific antibody molecules in which two antibody domains bind the HER2/*neu* tumor antigen, and one or two additional domains bind a chelate (CHX-A") that can be conjugated to a cytokine. We have successfully produced minibodies in various formats (see figure below). Moreover, anti-CHX-A" single-chain Fv molecules have been panned successfully from a human phage display library; we plan to clone these scFv into the minibody constructs to create bispecific molecules with the desired tumor pretargeting characteristics. When this has been accomplished will conjugate IL-2 to CHX-A" and examine the potential value of this pretargeting approach.

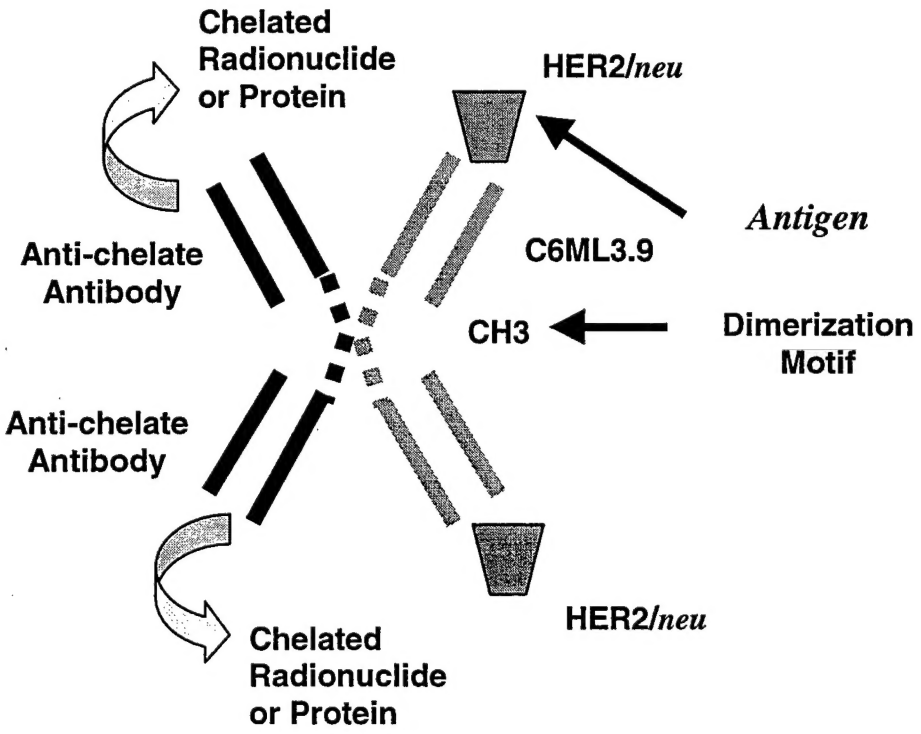



Figure 1. Design of New Pretargeting Vehicle

Thus far we have created an initial template containing antibody domains that we have extensively worked with in the past. One antibody contains anti-HER2/*neu* binding specificity and the other antibody has specificity for a different ligand, FcγRIII (which is the Fcγ receptor, CD16, with specificity for Fc domains of immunoglobulin G (Figure 2).

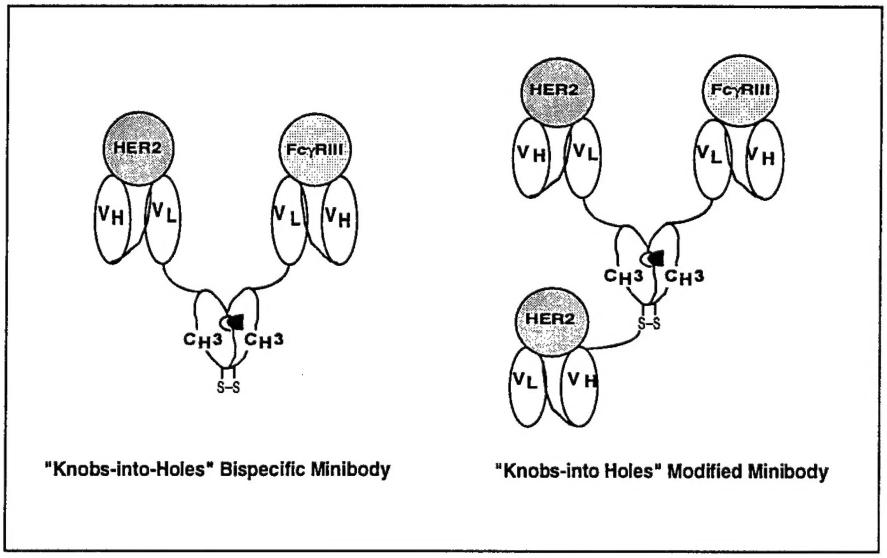
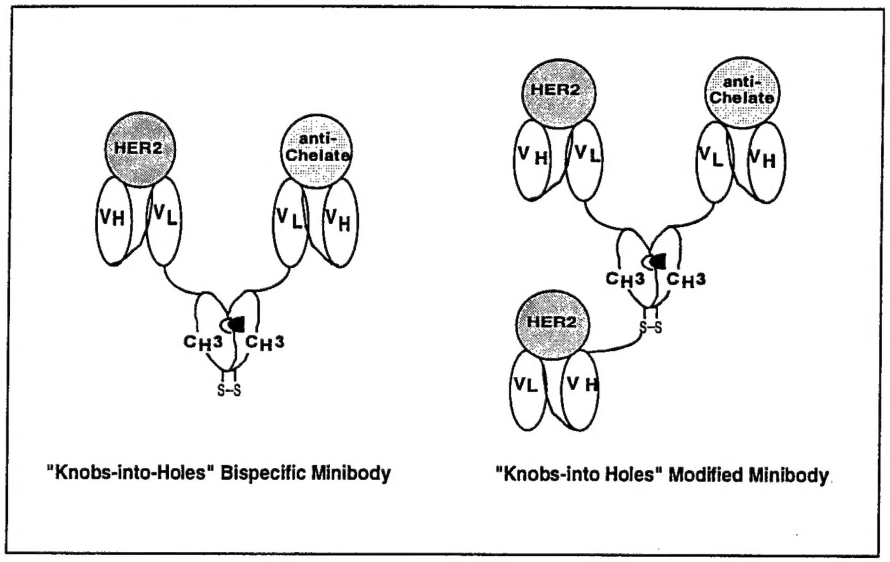



Figure 2. Schematic depicting the Structure of the two distinct minibody structures

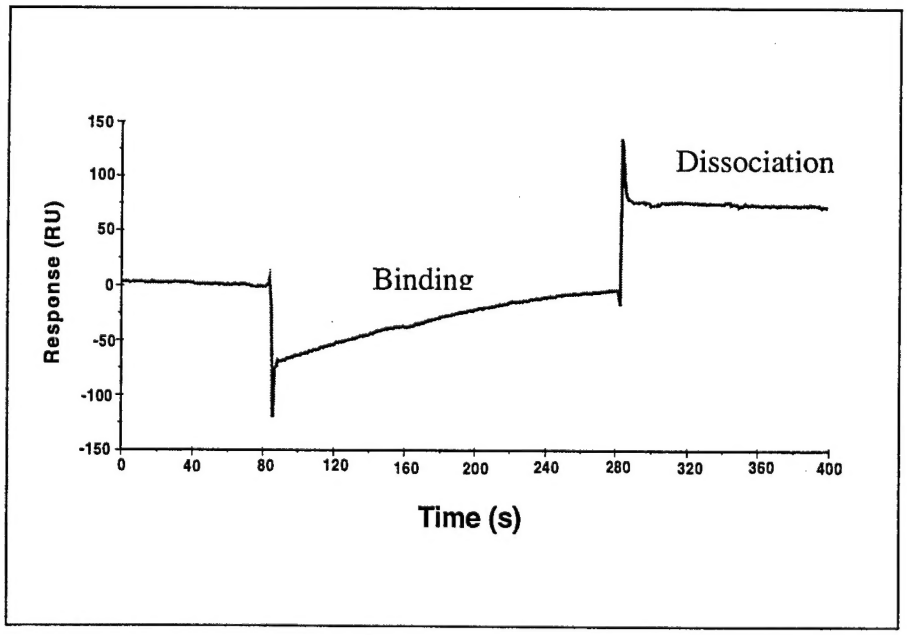
As is evident in this figure, both structures employ the "knobs-into-holes" concept, whereby heterodimerization based on affinities of CH3 domains is favored. Two distinct bispecific minibodies have been produced. The first is a dimeric molecule (left figure), while the other is trimeric, as depicted on the right side of the figure. Tetramers can be produced as well (not shown). Since the genes for these molecules are in hand, it is relatively straightforward to excise the anti-FcγRIII variable heavy and light chain genes and replace them with $V_H$ and $V_L$ genes encoding protein domains that bind to the metal chelate, CHX-A". To that end, we are obtaining single-chain Fv molecules that bind to this chelate from a long-time collaborator, Dr. James Marks and intend to create bispecific minibodies that bind to HER2/*neu* and to CHX-A" (see Figure 3 below).

Figure 3. Bispecific Antibodies targeting HER2/*neu* and CHX-A"




We anticipate success in producing the bispecific minibodies schematized above. The anti-HER2/*neu* x anti-FcγRIII minibodies exhibit the desired binding properties in a plasmon sensor resonance assay (BIAcore, Pharmacia). Shown below (Figure 4) is a representative tracing employing the dimeric bispecific minibody (Figure 2, left).

Figure 4. Anti-HER2/*neu* binding and slow dissociation of a dimeric, bispecific minibody



Similar results were demonstrated for binding to FcγRIII (not shown).

A novel "sandwich" based BIAcore assay was developed to investigate the capacity of the minibody to mediate bispecific binding. Figure 5 and Table 1 below show that the minibody, but not a monospecific antibody, was able to simultaneously bind to HER2/*neu* and to FcγRIII. Thus, these data support the idea that bispecific proteins can be produced and shown to mediate the desired binding properties. When the anti-CHX-A" variable heavy and light chain genes have been introduced into these structures, we intend to employ these molecules in tumors that overexpress HER2/*neu*; we have extensive experience with SK-OV-3 and with the breast cancer line MDA-MB-231-DYT2; both lines grow well in immunodeficient mice and express abundant HER2/*neu* on the cell surface.

Figure 5. Sandwich BIAcore assay demonstrates binding properties of a bispecific minibody

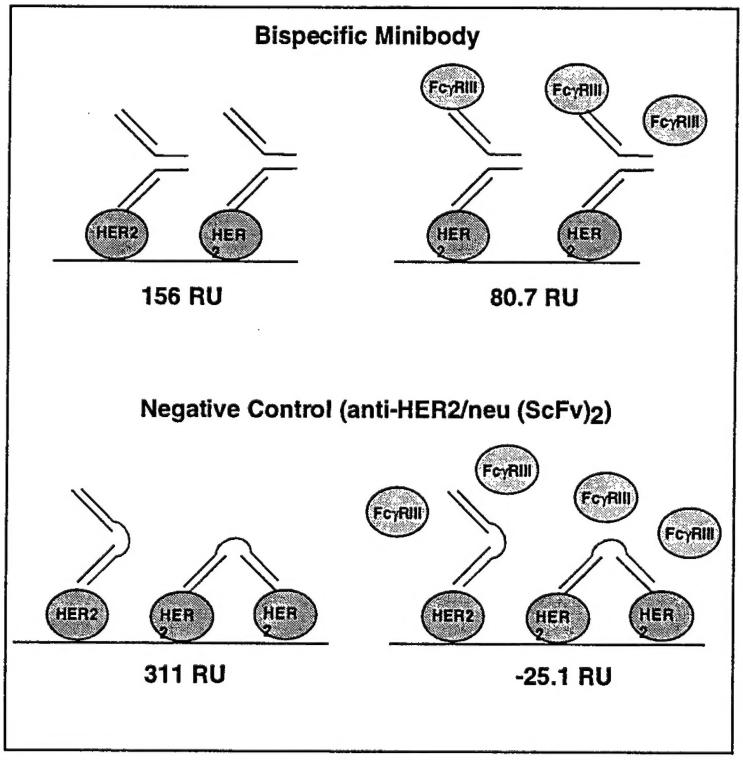


Table 1. Sandwich assay demonstrates bispecific binding properties of anti-HER2/*neu* x anti-FcγRIII minibody

| Structure | HER2 (RU) | CD16 (RU) | Bispecific Binding |
|---|---|---|---|
| YCMC | + (75) | + (98) | + |
| TriBi | + (154) | + (310) | + |
| KHYCMC | + (635) | + (162) | + |
| KHTriBi | + (440) | + (242) | + |
| Neg. Control | - | - | - |

We intend to modify IL-2 by covalently adding a labeled metal species (probably Indium-111) to its carboxyl terminus using standard technology extensively employed in our laboratory. The ability of the labeled IL-2 to be selectively retained at antibody-pretargeted tumor sites then will be determined.

## KEY RESEARCH ACCOMPLISHMENTS

1. Biotinylation of interleukin-2 (IL-2).

2. Characterization of biotinylated IL-2 binding properties, T-cell activation properties and capacity to promote lymphocyte-mediated cytotoxicity of tumor cells.

3. Demonstration that admixture of streptavidin-conjugated antibody with biotinylated IL-2 is associated with retention of antibody binding and IL-2 binding properties.

4. Proof of concept that antibody pretargeting can promote some selective tumor retention of IL-2.

5. Development of new vehicles for antibody-directed pretargeted cytokine therapy.

## REPORTABLE OUTCOMES

L. Shahied, R.K. Alpaugh, G.P. Adams, H.H. Simmons, E.M. Horak, C.C. Shaller, D.B. Axworthy, A.R. Amoroso, and L.M. Weiner (2000) Pretargeting Mechanism for the Delviery of Interleukin-2 to Tumor Site. The 11th Annual International Conference on Antibody Engineering (Poster Presentation).

## CONCLUSIONS

The results to date warrant continuation of this line of research. The new direction of the research results from a thorough analysis that showed the particular pretargeting strategy employed to date was not likely to yield successful IL-2 targeting and therapy. The new strategy builds directly upon other work in our laboratory and is felt to be a more promising avenue of research. Preliminary data demonstrate the feasibility of the proposed work.

## REFERENCES


1. Rosenberg SA, Lotze MT, Muul LM et al. A progress report on the treatment of 157 patients with advanced cancer using lymphokine activated killer cells and interleukin-2 or high dose interleukin-2 alone. New England Journal of Medicine 1987, 316: 889-97.
2. Mier JW, Aronson FR, Numerof RP, Vachino G, Atkins MB. Toxicity of immunotherapy with interleukin-2 and lymphokine-activated killer cells. Pathology & Immunopathology Research 1988, 7(6): 459-76.
3. Fyfe G, Fisher RI, Rosenberg SA, Sznol M, Parkinson DR, Louie AC. Results of treatment of 255 patients with metastatic renal cell carcinoma who received high-dose recombinant interleukin-2 therapy. Journal of Clinical Oncology 1995 13(3): 688-96.
4. Sparano JA, Fisher RI, Sunderland M, Margolin K, Ernest ML, Sznol M, Atkins MB, Dutcher JP, Micetich KC, Weiss GR et al. Randomized phase III trial of treatment with high-dose interleukin-2 either alone or in combination with interferon alfa-2a in patients with advanced melanoma. Journal of Clinical Oncology 1993, (10): 1969-77.
5. Talpaz M, Kantarjian H, Kurzrock R et al. Interferon-alpha produces sustained cytogenetic responses in chronic myelogenous leukemia. Philadelphia chromosome-positive patients. Annals of Internal Medicine 1991, 114(7): 532-8.
6. Vedantham S, Gamliel H, Golomb HM. Mechanism of interferon action in hairy cell leukemia: a model of effective cancer biotherapy. Cancer Research 1992, 52(5):1056-66.
7. Agarwala SS, Kirkwood JM. Interferons in melanoma. Current Opinion in Oncology 1996, (2): 167-74.
8. Kawakami Y, Eliyahu S,Jennings C et al. Recognition of multiple epitopes in the human melanoma antigen gp100 by tumor-infiltrating T lymphocytes associated with in vivo tumor regression. Journal of Immunology 1995, 154(8): 3961-8.
9. Levitsky HI, Lazenby A, Hayashi RJ et al. In vivo priming of two distinct antitumor effector populations: the role of MHC class I expression. Journal of Experimental Medicine 1994, 179(4): 1215-24.
10. Jaffee EM, Dranoff G, Cohen LK et al. High efficiency gene transfer into primary human tumor explants
without cell selection. Cancer Research 1993, 53(10 Suppl): 2221-6.
11. Del Prete G. The concept of type-1 and type-2 helper T cells and their cytokines in humans. International Reviews of Immunology 1998, 16(3-4): 427-55.
12. Golding B. Scott DE. Vaccine strategies: targeting helper T cell responses. Annals of the New York Academy of Sciences 1995, 754:126-37.
13. Tahara H. Lotze MT. Antitumor effects of interleukin-12 (IL-12): applications for the immunotherapy and gene therapy of cancer. Gene Therapy 1995, 2(2): 96-106.
14. Greenfield EA. Howard E. Paradis T. et al. B7.2 expressed by T cells does not induce CD28-mediated costimulatory activity but retains CTLA4 binding: implications for induction of antitumor immunity to T cell tumors. Journal of Immunology 1997, 158(5): 2025-34.
15. Noel PJ. Boise LH. Thompson CB. Regulation of T cell activation by CD28 and CTLA4. Advances in Experimental Medicine & Biology 1996, 406:209-17.

16. Zitvogel L, Robbins PD, Storkus WJ et al. Interleukin-12 and B7.1 co-stimulation cooperate in the induction of effective antitumor immunity and therapy of established tumors. European Journal of Immunology 1996, 26(6): 1335-41.
17. Wojtowicz-Praga S. Reversal of tumor-induced immunosuppression: a new approach to cancer therapy. Journal of Immunotherapy 1997, 20(3): 165-77.
18. Lode HN, Xiang R, Varki NM et al. Targeted interleukin-2 therapy for spontaneous neuroblastoma metastases to bone marrow. Journal of the National Cancer Institute 1997, 89(21): 1586-94.
19. Xiang R, Lode HN, Dolman CS et al. Elimination of established murine colon carcinoma metastases by antibody-interleukin 2 fusion protein therapy. Cancer Research 1997, 57(21): 4948-55.
20. Hornick JL, Khawli LA, Hu P et al. Chimeric CLL-1 antibody fusion proteins containing granulocyte-macrophage colony-stimulating factor or interleukin-2 with specificity for B-cell malignancies exhibit enhanced effector functions while retaining tumor targeting properties. Blood, 1997, 89(12): 4437-47.
21. Hu P, Hornick JL, Glasky MS et al. A chimeric Lym-1/interleukin 2 fusion protein for increasing tumor vascular permeability and enhancing antibody uptake. Cancer Research 1996, 56(21): 4998-5004.
22. Gallinger S, Reilly RM, Kirsh JC et al. Comparative dual label study of first and second generation antitumor-associated glycoprotein-72 monoclonal antibodies in colorectal cancer patients. Cancer Research 1993, 53(2): 271-8.
23. Bagshawe KD. Antibody-directed enzyme prodrug therapy (ADEPT). Advances in Pharmacology 1993, 24:99-121.
24. Goodwin DA, Meares CF, McCall MJ et al. Pretargeted immunoscintography of murine tumors with indium-11-labeled bifunctional haptens. Journal of Nuclear Medicine 1988, 29: 226-34.
25. Paganelli G, Riva P, Deleide G et al. In vivo labeling of biotinylated antibodies by radioactive avidin: a strategy to increase tumor radiolocalization. International Journal of Cancer 1988, 2 (suppl):121-25.
26. Axworthy DB, Beaumier PL, Bottino BJ et al. Preclinical optimization of pretargeted radioimmunotherapy components: High efficiency curative $^{90}Y$ delivery to mouse tumor xenografts. Tumor Targeting 2: 156-157, 1996.
27. Krigel RL, Padavic-Shaller KA, Rudolph AA et al. Hemorrhagic gastritis as a new dose-limiting toxicity of recombinant tumor necrosis factor. Journal of the National Cancer Institute 1991, 83(2): 129-31.
28. Nanni P, De Giovanni C, Landuzzi L et al. Therapy of murine mammary carcinoma metastasis with interferon gamma and MHC gene-transduced tumour cells. British Journal of Cancer 1996, 74(10): 1564-9.
29. Yanagihara K, Seyama T, Watanabe Y et al. Antitumor potential of interferon-gamma: retroviral expression of mouse interferon-gamma cDNA in two kinds of highly metastatic mouse tumor lines reduces their tumorigenicity. Natural Immunity 1994,13(2-3): 102-12.
30. Esche C, Subbotin V, Maliszewski C et al. FLT3 ligand administration inhibits tumor growth in murine melanoma and lymphoma. Cancer Research 1998, 58(3): 380-3.

31. Lynch DH, Andreasen A, Maraskovsky E et al. Flt3 ligand induces tumor regression and antitumor immune responses in vivo. Nature Medicine 1997, 3(6): 625-31.
32. Campbell JJ, Qin S, Bacon KB et al. Biology of chemokine and classical chemoattractant receptors:
differential requirements for adhesion-triggering versus chemotactic responses in lymphoid cells. Journal of Cell Biology 1996, 134(1): 255-66.
33. Falk W, Leonard EJ. Human monocyte chemotaxis: migrating cells are a subpopulation with multiple chemotaxin specificities on each cell. Infection & Immunity 1980, (3): 953-9.
34. Ember JA, Sanderson SD, Hugli TE et al. Induction of interleukin-8 synthesis from monocytes by human C5a anaphylatoxin. American Journal of Pathology 1994,144(2): 393-403.
35. Adams GP, McCartney JE, Tai M-S, Oppermann H, Huston JS, Stafford III WF, Bookman MA, Fand I, Houston LL, Weiner LM. Highly specific in vivo tumor targeting by monovalent and divalent forms of 741F8 anti-c-erbB-2 single-chain Fv. Cancer Res 1993, 53:4026-4034.
36. Weiner LM, Clark JI, Davey M, Li WS, Garcia de Palazzo I, Ring DB, Alpaugh RK. Phase I trial of 2B1, A bispecific monoclonal antibody targeting c-erbB-2 and FcγRIII. Cancer Res 1995, 55:4586-4593.
37. Weiner LM, Holmes M, Richeson A, Godwin A, Adams GP, Hsieh-Ma ST, Ring DB and Alpaugh RK. Binding and Cytotoxicity Characteristics of the Bispecific Murine Monoclonal Antibody 2B1. J Immunology 1993, 151:1-9.
38. Shpitz B, Chambers CA, Singhal AB, Hozumi N, Fernandes BJ, Roifman CM, Weiner LM, Roder JC, Gallinger S. High level functional engraftment of severe combined immunodeficient mice with human peripheral blood lymphocytes following pretreatment with radiation and anti-asialo $GM_1$. J Immunol Methods 1994, 169:1-15.
39. Weiner LM, Holmes M, Adams GP, LaCreta F, Watts P, de Palazzo IG. A Human Tumor Xenograft Model of Therapy with a Bispecific Monoclonal Antibody Targeting c-erbB-2 and CD16. Cancer Res. 53:94-100, 1993.
40. Stone D, Axworthy D, Sanderson J, Graves S, Reno J. Pretargeting as a delivery system for tumor-specific localization of TNF-alpha. Proceedings Amer Assoc Cancer Res 1996, 37(A40):484.